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Award Number: W81XWH-07-1-0084

TITLE: Design of an NF-kB Activation-Coupled Apoptotic Molecule for Prostate Cancer Therapy

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REPORT DATE: July 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31-07-2008	Final	1 JAN 2007 - 30 JUN 2008

4. TITLE AND SUBTITLE
Design of an NF-kB Activation-Coupled Apoptotic Molecule for Prostate Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0084

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Geisinger Clinic
Danville, PA 17822

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This proposal is to establish an effective and selective gene therapy technique for prostate cancer. The main idea is to create an NF-kB/Caspase-3 fusion protein, designated as NF-kB activation-coupled apoptotic molecule (NACAM), that couples activation of NF-kB to activation of Caspase-3, thus to apoptosis. During the eighteen months, we constructed 32 plasmids for testing the function of NACAM and determined the expression of the plasmids in HEK293 cells. We further tested the function of NACAM by examining the caspase activity of NACAM and interaction of NF-kB/Caspase-3 fusion proteins. We found that p65-L-SS and p65-L-LS are capable of forming active caspase-3. However, because NF-kB/Caspase-3 fusion proteins form homo-dimers, NF-kB/Caspase-3 subunit fusion proteins have only small portion to form NACAM. Nevertheless, our work provided a preliminary study for creating an NF-kB activation-coupled apoptosis molecule. In addition to NACAM, we continued our research on development of mitogenesis-coupled apoptosis molecular device (MCAMD). The first-phase research of MCAMD was funded by DOD-PCRP concept award (W81XWH-05-1-0178) and completed in June of 2006. Because MCAMD is technically related to NACAM, we further developed MCAMD with this funding support and successfully established MCAMD system in HEK293 cells. We have observed that mitogenic conditions induced cellular apoptosis through MCAMD, providing preliminary data for application of MCAMD to prostate cancer therapy. This work is in preparation of manuscript for publication and in process of application for a patent. Future studies will include: (a) to further characterize and improve NACAM; and (b) to test the function of MCAMD for killing cancer cells in a prostate cancer cell system and a prostate cancer cell-xenografted mouse model.

15. SUBJECT TERMS
NF-kB; Apoptosis; Caspase-3; Ras; Raf; Protein engineering

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	23	USAMRMC

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Introduction

NF-κB is a family of transcriptional factors which activity is highly regulated in response to multiple signals [1]. NF-κB family has five members: p65(RelA), RelB, c-Rel, p105/p50(NF-κB1), and p100/p52 (NF-κB2) [1]. A conserved 300 amino acid domain (Rel homology domain, RHD, Fig 1A) is located at the N-terminus of all the NF-κB family members. RHD domain is the region to form homodimers or heterodimers among the members, interact with IκBs, and bind to DNA. In inactivation status, NF-κB dimers are bound to IκB and incapable of interacting with DNA, thus have no transcriptional activity. Upon an activation signal, IκB kinase (IKK) is activated, and as a result, IκB is phosphorylated and ubiquitinated, and subsequently degraded by proteasomes [1]. The IκB-free NF-κB dimers are capable of binding to DNA and activating gene transcriptions.

NF-κB-activated gene transcription is critical for cell survival, thus plays key roles in tumorigenesis, chemotherapy- and radiotherapy- resistance, and tumor metastasis [2, 3]. NF-κB is constitutively active in androgen-independent prostate cancer cells [4, 5]. This project attempts to design a biomolecule that couples activation of NF-κB to apoptosis (cell death) for prostate cancer therapy that can selectively eliminate NF-κB-activated prostate cancer cells. To fulfill the goal, the NF-κB activation-coupled apoptotic molecule (NACAM) is constructed by fusing the large subunit (C3LS) and the small subunit (C3SS) of Caspase-3 separately to p65 NF-κB. In cells, p65-C3LS and p65-C3SS form dimers. When NF-κB signaling is inactive, the p65-C3LS/p65-C3SS dimer is bound to IκB, the C3LS and the C3SS cannot access to each other, thus cannot form mature Caspase-3. When NF-κB signaling is active, IκB is degraded and the p65-C3LS/p65-C3SS dimer is IκB-free, thus the C3LS and the C3SS become accessible to each other and form a mature active Caspase-3 to induce cellular apoptosis. Therefore, the p65-C3LS/p65-C3SS dimer couples activation of NF-κB to activation of Caspase-3 and cellular apoptosis. Introducing the p65-C3LS/p65-C3SS dimer into androgen-independent prostate cancer cells, in which NF-κB signaling is constitutively active, is expected to generate active Caspase-3 from the NACAM (p65-C3LS/p65-C3SS) and subsequently induce cellular apoptosis in the cancer cells. By this approach, we will selectively kill NF-κB-activated cancer cells and control NF-κB-activated tumor occurrence and recurrence.

Body

1. NF-κB activation-coupled apoptosis molecule (NACAM).

1.1. Construction of plasmids that are required for testing NF-κB activation-coupled apoptosis. The p65 NF-κB (RelA) is a ubiquitously expressed isoform that is also present in prostate cancer cells [2, 3]. The structure model of NF-κB p65 has been established (Fig 1) [6, 7]. Thus, we focused on construction and characterization of p65-based NACAM. In addition, we also used NF-κB p50 and NF-κB p105 to construct the NACAM for supportive experiments.

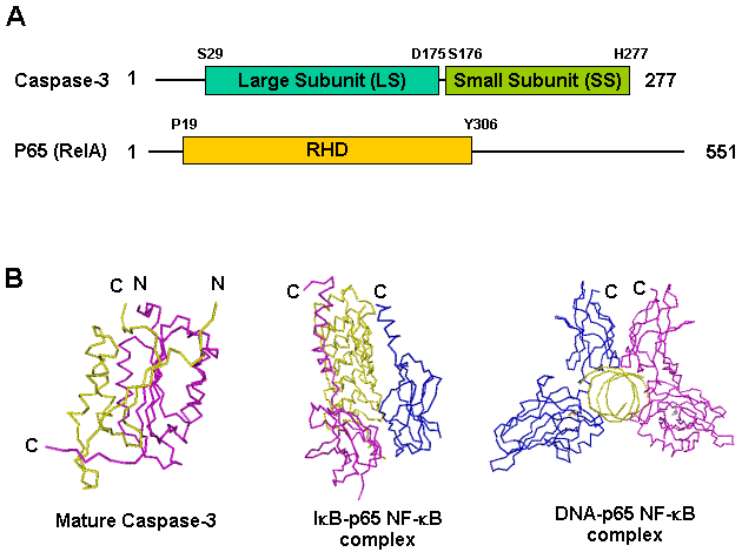

Figure 1 The structure of Caspase-3 and p65 NF-κB. ***A,*** the schematic representation of pro-Caspase-3 and p65 NF-κB. The numbers indicate amino acid residue positions. The letter before the number indicates the amino acid at the position. In p65 NF-κB, RHD, Rel homology domain. ***B,*** The three dimensional structure of mature Caspase-3 (the left panel, PDB ID: 1QX3), the complex of IκB and p65 NF-κB dimer (the middle panel, PDB ID:1OY3), and the complex of DNA and p65 NF-κB dimer (the right panel, PDB ID:1RAM). In the left panel, the yellow-colored peptide is the small subunit of Caspase-3 and the pink-colored peptide is the large subunit. In the middle and right panels, the blue-and pink-colored peptides are p65 NF-κB dimer. The yellow-colored peptide in the middle panel is IκB and the yellow-colored peptide in the right panel is DNA. The peptide termini are labeled with letter C or N. C, carboxyl terminus; N, amino terminus.

How to fuse the LS and the SS of Caspase-3 to p65 NF-κB is a key step in construction of a functional NACAM. The primary structure of pro-Caspase-3 and p65 NF-κB (RelA) is shown in Figure 1A. The pro-Caspase-3 contains two subunits of the mature Caspase-3 (the left panel, Figure 1B): the large subunit (LS) and the small subunit (SS). Two requirements need to be met for a

functional NACAM: (a) when NF-κB signal is inactivated, NACAM does not form active Caspase-3; (b) when NF-κB signal is activated, NACAM generates active Caspase-3. To construct p65 NF-κB/caspase-3 fusion proteins that can effectively form a NACAM, we referred the crystal structure of mature Caspase-3, inactive p65 NF-κB dimer (p65 NF-κB dimer complexed with IκB), and active p65 NF-κB dimer (p65 NF-κB dimer complexed with DNA), as shown in Figure 1B. We noticed following structural features of Caspase-3 and p65 NF-κB. (a) The amino terminus of large subunit of Caspase-3 is positioned closely to both the amino terminus and the carboxyl terminus of small subunit of Caspase-3 in the mature Caspase-3 (the left panel, Fig 1B). (b) The amino-termini of p65 NF-κB dimer in both inactive and active forms are far apart (the middle and right panels, Fig 1B). (c) The carboxyl termini of inactive p65 NF-κB dimer are separated by IκB (the middle panel, Fig 1B). (d) The carboxyl termini of active p65 NF-κB dimer are closely positioned (the right panel, Fig 1B). Therefore, we fused the amino terminus of the LS and the SS of Caspase-3 to the carboxyl terminus of p65 NF-κB. In this orientation, the IκB-free p65-LS/p65-SS heterodimer (active form) is likely to generate a mature active Caspase-3.

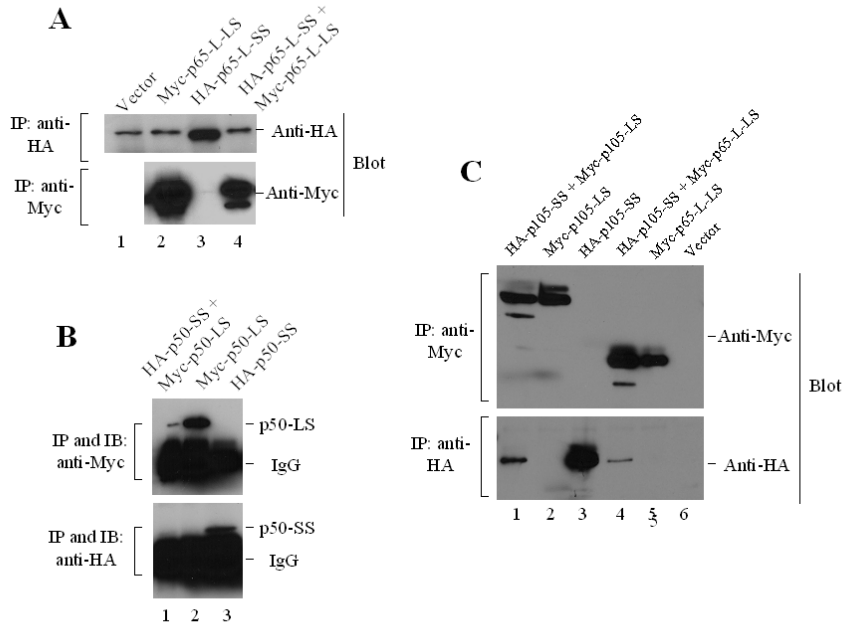

Figure 2. Expression of NF-κB/Caspase-3 fusion proteins. The tagged p65-L-SS, p65-L-LS, p50-SS, p50-LS, p105-SS, or p105-LS (2 μg/each) was transfected or co-transfected with the indicated plasmid in HEK293 cells for 48 hrs. The cells were lysed with the lysis buffer. The fusion proteins were immunoprecipitated and immunoblotted with anti-Myc or anti-HA antibody. A, Expression of p65-L-SS and p65-L-LS. B, Expression of p50-SS and p50-LS. C, Expression of p105-SS, p105-LS and p65-L-LS. L, linker; SS, small subunit of caspase-3; LS, large subunit of caspase-3.

To ensure a free rotation of the LS and the SS portion in the fusion proteins so that the LS and the SS are capable of forming active mature Caspase-3 in activated (IκB-free) p65-LS/p65-SS dimers, we added a flexible linker (GGGGS) between p65-NF-κB and the LS or the SS.

The engineering of the fusion proteins was performed by polymerase chain reaction (PCR)-directed mutagenesis. The cDNAs of human NF-κB and pro-Caspase-3 were used as the PCR templates. The flexible linker oligo that codes for (GGGGS) was incorporated into the cDNA of the fusion proteins by PCR. Two expression vectors were used for construction of the fusion proteins. One is pCDNA3 that was used for constitutive expression of the engineered proteins; the other is tetracycline inducible expression vector pTet (Invitrogen) that is capable of controlling expression level of the fusion proteins. To inactivate NF-κB/Caspase-3 fusion protein dimers, IκBα was used for co-expression with NF-κB/Caspase-3 fusion proteins. IκBα was cloned into pcDNA3 vector with triple flag-tag. We tagged NF-κB-C3LS fusion proteins with Myc-tag, and NF-κB-C3SS fusion proteins with HA-tag and IκBα with HA-tag for convenience of immunoprecipitation and immunoblotting of the proteins.

Total 32 plasmids were constructed for the experiments. The plasmids can be classified into two groups: (a) The plasmids that were used for directly testing function of NACAM. These plasmids are: pCDNA3.1-myc-p65-L-C3LS; pCDNA3.1-HA-p65-L-C3LS; pCDNA3.1-HA-p65-link-C3SS; pCDNA3-myc-p65-C3SS; pCDNA3-myc-p65-C3LS; pTet-myc-p65-L-C3LS; pTet-myc-p65-C3LS; pTet-HA-p65-L-C3SS; pTet-HA-p65-C3SS; pCDNA3-myc-p105-L-C3LS; pCDNA3-myc-p105-C3LS; pCDNA3-HA-p105-L-C3SS; pCDNA3- HA-p105-C3SS; pCDNA3-myc-p50-L-C3LS; pCDNA3-myc-p50-L-C3SS; pCDNA3-myc-p50-C3LS; pCDNA3-HA-p50-C3SS; pTet-myc-p50-L-C3LS; pTet-HA-p50-L-C3SS; pTet-myc-p50-C3LS; pTet-HA-p50-C3SS p3XLFlag7.1-IκB alpha. (b) The plasmids that were used for controls. These plasmids are: pCDNA3-HA- p65; pCDNA3-myc-p105; pCDNA3-HA-C3LS; pCDNA3-HA-C3SS; pEGFPC1-C3SS; pCDNA3-myc- p50; pTet-myc-p50; pTet-HA-Caspase 3-C3LS; pTet-HA-Caspase 3-C3SS; pTet- myc-p105-C3LS. In the names, L stands for a flexible linker (GGGGS); C3LS for the large subunit of caspase-3; C3SS for the small subunit of caspase-3.

1.2. Results of testing NACAM. First, we examined the expression of the constructed plasmids in HEK293 cells. After 48 h transfection of the plasmids in HEK293 cells, we immunoprecipitated and immunoblotted expressed proteins with anti-Myc, anti-HA, anti-GFP or anti-flag antibody. We had successfully expressed all the proteins constructed. To test whether the constructs can form effective NACAM, we co-transfected NF-κB-SS with NF-κB-LS in HEK293 cells. As shown in Figure 2, p65-L-SS, p65-L-LS, p50-SS, p50-LS, p105-SS, or p105-LS alone was expressed well in cells (lanes 2 and 3 in Fig. 2A, 2B, and 2C). However, when co-transfected of p65-

L-SS with p65-L-LS (lane 4 in Fig 2A) or p50-SS with p50-LS (lane 1 in Fig 2B), expression of p65-L-SS or p50-SS was diminished, and expression of p65-L-LS or p50-LS was dramatically reduced. These data suggest that co-expression of p65-L-SS with p65-L-LS or p50-SS with p50-LS may induce cellular apoptosis that lowered expression level of the proteins. The expression of p105-SS and p105-LS when co-transfected was comparable to the expression level of the proteins when transfected alone (lane 1 in Fig 2C). This suggests that p105-SS and p105-LS may not form an effective NACAM, possibly due to conformation hindrance to interaction between LS and SS of caspase-3 in the fusion proteins that is required for formation of mature caspase-3 upon p105 dimer activation.

Next we tested whether p65-L-SS/p65-L-LS can form NACAM, i.e. p65-L-SS/p65-L-LS dimer has caspase activity. Because p65-L-SS/p65-L-LS cannot co-express, we took a different approach to acquire the p65-L-SS/p65-L-LS dimer. We expressed HA-tagged p65-L-SS or p65-SS (no linker) and Myc-tagged p65-L-LS or p65-LS (no linker) separately in HEK293 cells. After lysis of the cells, we combined the two cell lysates and let p65-L-SS (or p65-SS) interact with p65-L-LS (or p65-LS), then immunoprecipitated HA-p65-L-SS or p65-SS with anti-HA antibody. This immuno-complex would be a mixture of HA-p65-L-SS (or p65-SS) homo-dimer and HA-p65-L-SS/Myc-p65-L-LS (or p65-SS/p65-LS) hetero-dimer. We used this immunocomplex for caspase activity assay using a colorimetric caspase activity assay kit (Biovision). The purified Caspase-3 (1 unit) was used as a positive control in the assay. At the same time, we setup a series of control samples in the assay including small subunit (SS) and large subunit (LS) of caspase-3, p105-SS and p105-LS, and vector. As shown in Figure 3, we observed a measurable caspase-3 activity in the

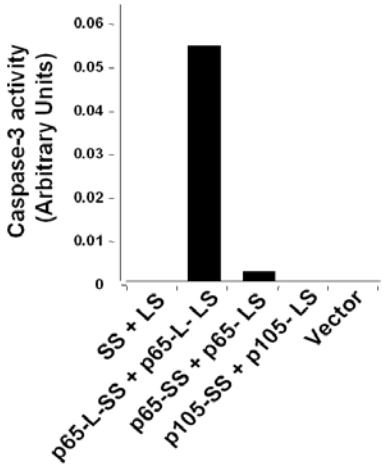

Figure 3. p65-L-SS/p65-L-LS are capable of forming active caspase-3. HA-p65-L-SS, HA-p65-SS, HA-p105-SS, HA-LS (caspase-3 large subunit), Myc-p65-L-LS, Myc-p65-LS, Myc-p105-LS, GFP-SS(caspase-3 small subunit) or pcDNA3 (vector) was transfected into HEK293 cells for 48 hrs. After lysis of the cells, the cell lysates containing the small subunit (SS) or SS-fusion proteins were mixed with the lysates containing the large subunit (LS) or LS-fusion proteins as indicated in the figure. The HA-tagged proteins were immunoprecipitated with anti-HA antibody and used for caspase-3 activity assay. The assay was carried out at 22 C for 2 hrs and the activity was measured and calculated based on comparison with a positive control using purified caspase-3 (1 unit).

mixture of p65-L-SS/p65-L-LS, an insignificant caspase activity in the mixture of p65-SS/p65-LS, and no caspase activity in the mixture of p105-SS/p105-LS or SS/LS. These data indicate that (a) separated small subunit and large subunit of caspase-3 cannot form a mature caspaes-3; (b) p65-L-SS/p65-L-LS can form active caspase-3; (c) a linker between p65 and caspase subunit in the fusion protein is important for formation of active caspase-3; and (d) p105-SS/p105-LS cannot form active caspase-3, which is consistent with the expression profile in Fig 2.

Because caspase-3 activity of the p65-L-SS/p65-L-LS mix was low comparing to that of positive control, we suspected that most of anti-HA-immunocomplex was HA-p65-L-SS homo-dimer. Only very small portion was p65-L-SS/p65-L-LS hetero-dimer. To confirm this, we used HA-p65 instead of HA-p65-L-SS to determine the dimer formation. We designed two experiments: the first one is expression of HA-p65 and Myc-p65-L-LS separately followed by mixing the both lysates for anti-HA immunoprecipitation; the second one is co-expression of HA-65 with Myc-p65-L-LS followed by anti-HA immunoprecipitation. The results are shown in Figure 4. Because interaction between p65 and p65-L-LS would not produce caspase activity and cause apoptosis, both p65 and p65-L-LS were very well expressed either in co-expression (lanes 1 and 3, Fig 4A) or separate expression (lanes 2 and 4, Fig 4A). We performed immunoprecipitation with anti-HA antibody in p65/p65-L-LS co-expressed lysates (lane 1, Fig 4B) or the mixture of p65-expressed and p65-L-LS-expressed lysates (lane 2, Fig. 4B). Co-immunoprecipitated Myc-p65-L-LS was detected by immunoblotting the anti-HA-immuno-complex with anti-Myc antibody (the upper panel, Fig 4B). Figure 4B clearly indicates that co-expression of p65/p65-L-LS yielded much more p65/p65-L-LS hetero-dimer (lane 1) than the mixture of the separate expression sample (lane 2). These data suggest that mixing the lysates containing

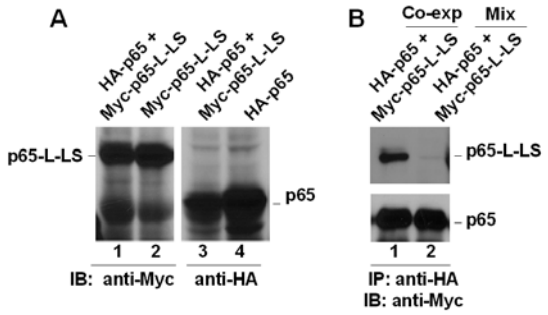

Figure 4. Co-expression of p65 with p65-L-LS produces more p65/p65-L-LS hetero-dimer than mixing the lysates from separate expression of p65 and p65-L-LS. HA-tagged p65 either co-transfected with or separately transfected from Myc-tagged p65-L-LS into HEK293 cells forr 48 hrs. The cell lysates containing p65 (lane 4 in **A**) was mixed with the lysates containing p65-L-LS (lane 2 in **A**). HA-tagged p65 was immunoprecipitated from either p65/p65-L-LS-co-transfected lysates or -mixed lysates. The co-immunoprecipitated Myc-p65-L-LS was detected by immunoblotting with anti-Myc antibody (the upper panel in **B**). **A**, the cell lysate samples; **B**, co-immunoprecipitation samples.

separately expressed p65-L-SS and p65-L-LS would not produce significant amount of hetero-dimer, probably due to formation of the homo-dimer of p65-L-SS or p65-L-LS during their expression.

To further determine that p65-L-SS/p65-L-LS can form an effective NACAM that couples activation of NF-κB to activation of caspase-3 and cellular apoptosis, we have to co-express p65-L-SS with p65-L-LS. However, constitutive co-expression of these two fusion proteins was not seen as we have shown in Figure 2. To overcome this problem, we subcloned Myc-p65-L-LS or Myc-p105-LS (as a control) into a tetracycline-controlled (Tet-off) expression vector pTet. We co-transfected pTet-Myc-p65-L-LS or pTet-Myc-p105-L-LS and pTet-tTAK (for expression of tetracycline transactivator tTA) with pcDNA3-HA-p65-L-SS into HEK293 cells in presence of tetracycline for inhibition of expression of p65-L-LS or p105-LS. After 48 hrs, we induced expression of p65-L-LS or p105-LS by removing tetracycline from the culture medium for indicated time in Figure 5. Myc-p65-L-LS, Myc-p105-LS or HA-p65-L-SS was immunoprecipitated from the cell lysates with anti-Myc or anti-HA antibody, and detected by immunoblotting with anti-Myc or anti-HA antibody (Fig 5). Induction of expression of p65-L-LS or p105-LS after 16 hrs suppressed expression of p65-L-SS. Because p105-LS/p65-L-SS should not form active caspase-3 and cause cellular apoptosis, the suppression of expression of p65-L-SS by overexpression of p105-LS might be due to expression competition (transcriptional or/and translational competition). Maximal co-expression of p65-L-LS and p65-L-SS occurred between 4-8 hrs induction of expression of p65-L-LS (lanes 8 and 9, Fig 5), while maximal co-expression of p105-LS and p65-L-SS occurred between 8-16 hrs induction. To determine caspase activity in the cells, we used cleavage of Poly (ADP-ribose) Polymerase (PARP), which is a known substrate for caspase-3 [8],

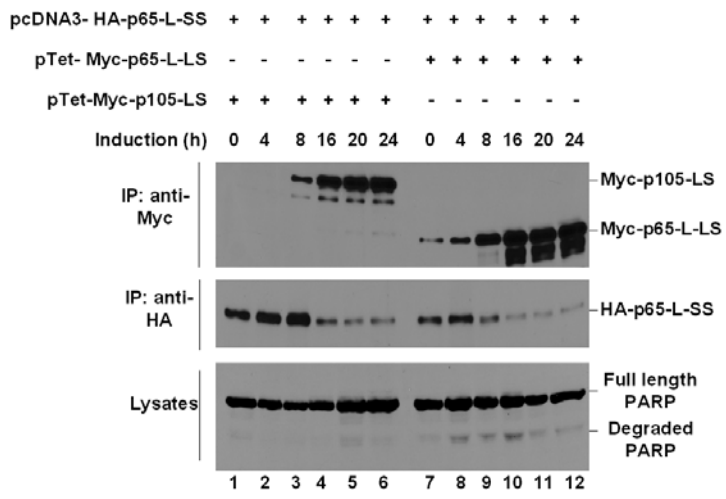

Figure 5. Expression of p65-L-LS and p105-LS by tetracycline-inducible expression system. pTet-Myc-p65-L-LS or pTet-Myc-p105-LS and pTet-tTAK were co-transfected with pcDNA3-HA-p65-L-SS into HEK293 cells in presence of tetracycline (for suppression of the expression). After 48 hrs, tetracycline in the medium was removed to start induction of expression p65-L-LS and p105-LS for indicated time. The expressed proteins were immunoprecipitated and immunoblotted with the antibodies against the tags (the top and middle panels). Cleavage of Poly (ADP-ribose) Polymerase (PARP) was used to indicate caspase-3 activity (the bottom panel).

to evaluate formation of active caspase-3 by p65-L-LS and p65-L-SS. As shown in Figure 5, co-expression of p65-L-LS and p65-L-SS caused a significant cleavage of PARP (lanes 8, 9, and 10), while co-expression of p105-LS and p65-L-SS did not show significant cleavage of PARP (lanes 3, 4, and 5), suggesting that p65-L-LS/p65-L-SS can form active caspase-3.

2. Ras/Raf/Caspase-3-based mitogenesis-coupled apoptosis molecular device (RRC-MCAMD).

2.1. Mitogenesis-coupled Apoptosis Molecular Device (MCAMD). MCAMD is a molecular device that senses tumorigenesis by monitoring cellular mitogenic process and induces apoptosis once cellular mitogenesis occurs. MCAMD contains two parts: one is tumorigenesis sensor portion and the other is death execution portion. Ras/Raf/caspase-3 (RRC)-based MCAMD is to couple the interaction of Ras and Raf to activation of caspase-3 and cellular apoptosis. The main idea of RRC-MCAMD is to fuse one subunit of caspase-3 to Raf and the other subunit to Ras. Once Ras interacts with Raf in response to mitogenic signal, the two subunits of caspase-3 that are fused to Ras and Raf will be brought together to form mature active caspase-3, thus cellular apoptosis will be initiated.

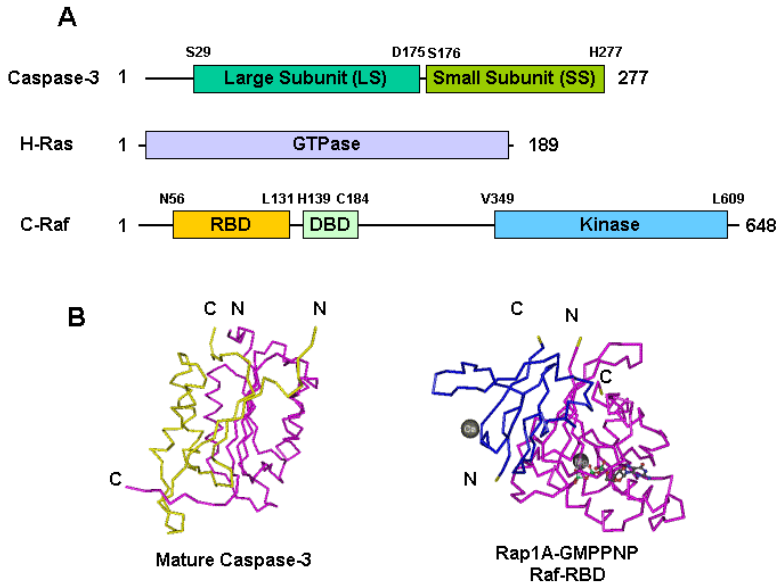

Figure 6 The structure of Caspase-3, Ras and Raf. ***A,*** the schematic representation of Caspase-3, H-Ras and C-Raf. The numbers indicate amino acid residue positions. The letter before the number indicates the amino acid at the position. In Raf, RBD, Ras-binding domain; DBD, DAG-binding domain. ***B,*** The three dimensional structure of mature caspase-3 (the left panel, PDB ID: 1QX3) and the complex of Ras family protein Rap1A and Raf-RBD (the right panel, PDB ID: 1C1Y). In the left panel, the yellow-colored peptide is the small subunit of caspase-3 and the pink-colored peptide is the large subunit. In the right panel, the blue-colored peptide is the Raf-RBD and the pink-colored peptide is Rap1A. The GTP analogue GMPPNP in Rap1A is labeled with rainbow colors. The metal ions are labeled with gray color. The peptide termini are labeled with letter C or N. C, carboxyl terminus; N, amino terminus.

We completed the first stage of research on the RRC-MCAMD with support of the Concept Award of Congressional-directed Medical Research Program-Prostate Cancer Research Program (CDMRP-PCRP) (award number: W81XWH-05-1-0178). We continued the research of RRC-MCAMD with the support of this grant. The work in **Sections 2.2 and 2.3**, which is described here to

help for understanding the continued research, was done with the previous grant support (award W81XWH-05-1-0178).

2.2. Construction of the RRC-MCAMD. We constructed the RRC-MCAMD based on following designs: we used Raf-RBD (the Ras-binding domain of Raf) instead of full length of Raf to make the fusion protein. In order to engineer appropriate structure of the fusion proteins so that the binding of Ras to Raf-RBD enables the fused LS and SS have correct orientation to form mature active caspase-3, we referred the conformational structure of the complex of Ras/Raf-RBD and mature caspase-3 from NCBI 3D structure database. Figure 6B shows the three-dimensional structure of mature caspase-3 and the complex of Ras family protein Rap1A and Raf-RBD that is extracted from NCBI structure database. The structure of mature caspase-3 (the left panel of Fig. 6B, PDB ID: 1QX3) indicates that the amino terminus of the LS is aligned with both the carboxyl and amino termini of the SS. The structure of the complex of Ras family protein Rap1A and Raf-RBD (the right panel, Fig. 6B, PDB ID: 1C1Y) shows that the carboxyl terminus of Raf-RBD is aligned with the amino terminus of Rap1A. Given that (a) the two subunits of caspase-3 that fused in Ras and Raf must be at the same side upon Ras/Raf bind each other, and (b) the amino terminus of Ras is the only site that can be used for the engineering because the carboxyl terminus of Ras is required for the farnesylation, therefore, we fused the amino terminus of the large subunit (LS) of caspase-3 to the carboxyl terminus of Raf-RBD, and the carboxyl terminus of the small subunit (SS) of caspase-3 to the amino terminus of Ras. To reduce the conformational restrictions to caspase-3 subunits fused to Ras or Raf, we added a flexible linker between Ras and the SS or Raf and the LS.

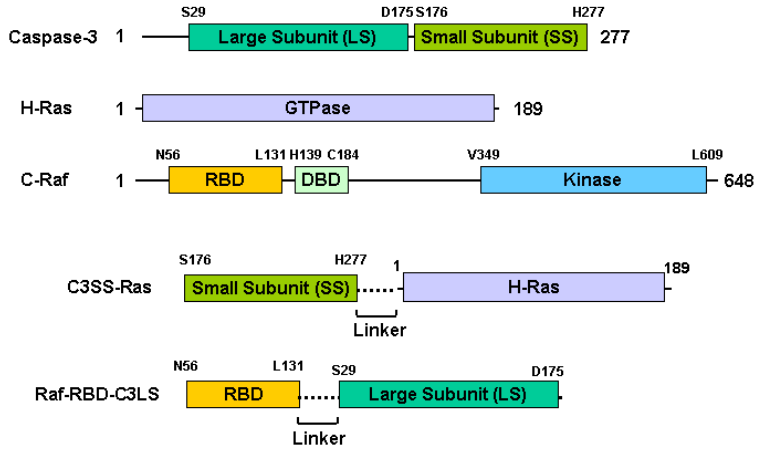

Figure 7. Construction of the RRC-MCAMD. The schematic representation of Caspase-3, H-Ras, C-Raf, C3SS-Ras and Raf-RBD-C3LS. The numbers indicate amino acid residue positions. The letter before the number indicates the amino acid at the position. In C3SS-Ras and Raf-RBD-C3LS, the amino acid residue positions are labeled according to the numbers in original proteins. In Raf, RBD, Ras-binding domain; DBD, DAG-binding domain.

The engineering of SS-Ras and Raf-LS fusion proteins was performed by polymerase chain reaction (PCR)-directed mutagenesis technique. The cDNA of human H-Ras, c-Raf and pro-caspase-3 was used as the PCR templates during the construction. The flexible linker $(GGGGS)_2$ was inserted between SS and Ras and the flexible linker (GNNGGNGGS) between Raf-BRD and LS. We tagged the fusion proteins with myc-tag or hemagglutinin (HA)-tag for the convenience of immunoprecipitation and detection of the fusion proteins. The constructs were shown in Figure 7.

2.3. Characterization of the RRC-MCAMD. After construction of the plasmids of the RRC-MCAMD, we tested the idea of MCAMD: coupling the interaction of Ras/Raf, a general mitogenic biochemical process, to Caspase-3 activation and apoptosis. The initial experiments were performed in COS7 cells for high efficiency of transfection and expression of engineered proteins.

The first question we addressed is whether the separated caspase-3 large subunit (C3LS) and small subunit (C3SS) are able to directly bind each other without the interaction of Ras and Raf . If so, then the RRC-MCAMD will not work. There are two methods for examination of the interaction: co-immunoprecipitation and GST-fusion protein pull-down. The co-immunoprecipitation, however, is not suitable for detection of a weak interaction. The GST-fusion protein pull-down can detect a very weak interaction due to excessive amount of fusion proteins. Therefore, we employed GST-fusion protein pull-down assay to examine direct interaction between the C3SS and C3LS. We incubated GST-C3LS-bound beads with C3SS-Ras-expressed COS7 cell lysates (right panel, Figure 8), and detected the amount of C3SS-Ras co-precipitated with GST-C3LS-bound beads. As shown in the left top panel of Figure 8, we did not detect any C3SS-Ras co-precipitated with GST-C3LS, indicating that no interaction between the C3SS and C3LS. These data suggest that separated C3SS and C3LS cannot form a mature Caspase-3 by themselves.

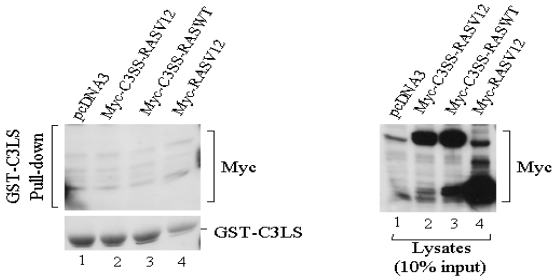

Figure 8. Separated C3SS and C3LS do not interact each other. GST-C3LS-bound beads were incubated with pcDNA3 (vector), Myc-tagged C3SS-RasG12V, C3SS-RasWT, and RasG12V transfected COS7 cell lysates (the right figure). The co-precipitated proteins were immunoblotted with anti-Myc (the left top panel). The loaded bead-bound GST-C3LS is shown in the left bottom panel by coomassie blue staining.

We next tested the interaction of Ras and Raf-RBD. When we co-expressed Myc-tagged Ras GTPase defective mutant G12V with HA-tagged Raf-RBD-C3LS, we observed interaction of RasG12V with Raf-RBD-C3LS using co-immunoprecipitation assays, as we expected (lane 2, Figures 9A and 9B). This indicates that the RBD domain of Raf is functional and can binds to Ras. However, when we co-expressed Myc-tagged C3SS-RasG12V with HA-tagged Raf-RBD-C3LS, we did not observe any expression of the two proteins in cell lysates (lane 1, the bottom two panels, Figure 9A and 9B). This suggests that co-expression of C3SS-RasG12V and Raf-RBD-C3LS induced a rapid cell death, thus no expression of either protein could be detected. To ensure that expression of C3SS-RasG12V or Raf-RBD-C3LS did not have any defect, we expressed these two plasmids separately in COS7 cells. As shown in Figure 9C, the expression of Myc-C3SS-RasG12V or HA-Raf-RBD-C3LS alone was normal (lanes 1 and 3, figure 9C), while no co-expression of these two fusion proteins was detected (lane 2, figure 9C). These data strongly indicate that C3SS-RasG12V and Raf-RBD-C3LS cannot co-exist in cells, implicating apoptosis and rapid cell death occurred upon co-expression of C3SS-RasG12V and Raf-RBD-C3LS.

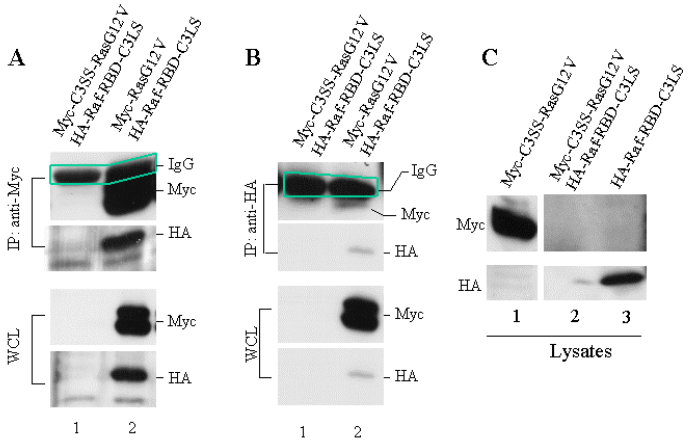

Figure 9 C3SS-RasG12V and Raf-RBD-C3LS cannot co-express in cells. Myc-tagged C3SS-RasG12V, RasG12V and HA-tagged Raf-RBD-C3LS were transfected or co-transfected into COS7 cells. Myc-tagged C3SS-RasG12V or RasG12V was immunoprecipitated by anti-Myc. HA-tagged Raf-RBD-C3LS was immunoprecipiated by anti-HA. The co-immunoprecipitated Raf-RBD-C3LS was detected by immunoblotting with anti-HA (the second top panel in Figure **A**) and co-immunoprecipitated Myc-RasG12V was detected with anti-Myc (the top panel of Figure **B**). The expression of the proteins was shown in whole cell lysates (WCL) (Figure **C** and the two bottom panels in figures **A** and **B**).

2.4. Binding of C3SS-RasG12V to Raf-RBD-C3LS yields an active caspase. To confirm that C3SS-RasG12V and Raf-RBD-C3LS are able to form a functional MCAMD, we transfected and expressed pcDNA3-Myc-RasG12V, pcDNA3-Myc-C3SS-RasG12V, pcDNA3-Myc-C3SS-Ras and pcDNA3-HA-Raf-RBD-C3LS separately into HEK293 cells. After lysis of the cells, we mixed the lysates containing Myc-RasG12V, Myc-C3SS-RasG12V, or Myc-C3SS-Ras with the lysates containing HA-Raf-RBD-C3LS, and immunoprecipitated HA-Raf-RBD-C3LS with an anti-HA antibody. The immunoprecipitation complex was subsequently used for Caspase-3 activity assay on an

artificial Caspase-3 substrate, DEVD-pNA (pNA: p-nitroalanine), by a Caspase-3 colorimetric assay kit (BioVision). The purified Caspase-3 (10 ng) was used as a positive control in the assay. As shown in Figure 10A, The immunoprecipitation from lysates containing Myc-C3SS-RasG12V and HA-Raf-RBD-C3LS had a caspase-3 activity comparable to the positive control Caspase-3, while the immunoprecipitation from the lysates containing Myc-C3SS-Ras and HA-Raf-RBD-C3LS had very little Caspase-3 activity, which was 10 folds lower than that with C3SS-RasG12V, indicating that binding of Raf-RBD-C3LS to C3SS-RasG12V assembles an active Caspase-3. As wild type Ras has very weak binding capacity to Raf-RBD, very little C3SS-Ras was co-immunoprecipitated with HA-Raf-RBD-C3LS, thus immunoprecipitation from the lysates containing HA-Raf-RBD-LS and Myc-C3SS-Ras attained little Caspase-3 activity. As expected, the negative control immunoprecipitation from the lysates containing Myc-RasG12V and HA-Raf-RBD-C3LS had no Caspase-3 activity (Figure 10A). Figure 10B shows the protein expression level, excluding the possibility that the difference in the caspase activity between samples results from the difference in protein expression level.

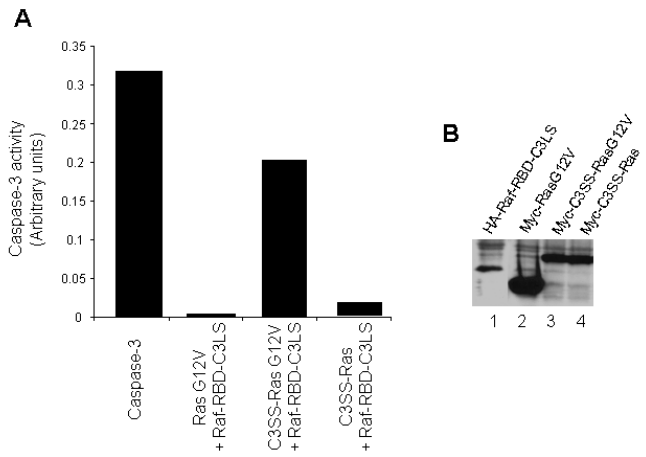

Figure 10. Binding of C3SS-RasG12V to Raf-RBD-C3LS yields an active Caspase-3. A, pcDNA3-HA-Raf-RBD-C3SS, pcDNA3-Myc-RasG12V, pcDNA3-Myc-C3SS-RasG12V, and pcDNA3-Myc-C3SS-Ras were transfected into HEK293 cells separately. After 48 hours transfection, the cells were lysed. The cell lysates that containing Myc-RasG12V, Myc-C3SS-Ras, or Myc-C3SS-RasG12V were mixed with the lysates containing HA-Raf-RBD-C3LS. The HA-Raf-RBD-C3LS was immunoprecipitated with an anti-HA antibody and the immunoprecipitates were used for Caspase-3 activity assay. The Caspase-3 activity assay was performed using a caspase-3 assay kit from BioVison. Purified mature Caspase-3 (10 ng) was used for a positive control. B, Expression level of the fusion proteins was detected by immunoblotting of the lysates with either anti-HA or -Myc antibody.

2.5. Mitogen stimulation activates caspase-3 activity of the RRC-MCAMD. To test if the caspase activity of the RRC-MCAMD is evoked by mitogenic signal, we employed a tetracycline-inducible expression system to control the expression of Myc-C3SS-Ras in cells. Myc-C3SS-Ras was cloned into a tet-off expression vector pTet-splice (Invitrogen), in which the expression of Myc-C3SS-Ras is induced by depleting tetracycline in culture medium. We co-transfected pTet-Myc-C3SS-Ras with pcDNA3-HA-Raf-RBD-C3LS or pcDNA3-HA-p65 (NF-κB)-C3LS (control) into HEK293 cells in presence of tetracycline. After 36 hours transfection, the cells were starved in a serum-free medium

for 12 hours. The expression of Myc-C3SS-Ras was initiated by removing the tetracycline in the medium for desired time. To generate a mitogenic signal, the cells were stimulated with 10% fetal bovine serum (FBS) for 4 hours. The HA-Raf-RBD-C3LS or HA-p65 (NF-κB)-C3LS (control) was immunprecipitated and used for Caspase-3 activity assay. As shown in Figure 11, with serum stimulation, the immunoprecipitated HA-Raf-RBD-C3LS had a significant caspase-3 activity, suggesting that HA-Raf-RBD-C3LS binds to serum-activated Myc-C3SS-Ras to generate an active caspase-3. Without serum stimulation, immunoprecipitated HA-Raf-RBD-C3LS had little caspase-3 activity. As expected, in the negative control sample, immunoprecipitated HA-p65(NF-κB)-C3LS did not have caspase-3 activity upon serum stimulation. These data indicate that the RRC-MCAMD is functioning.

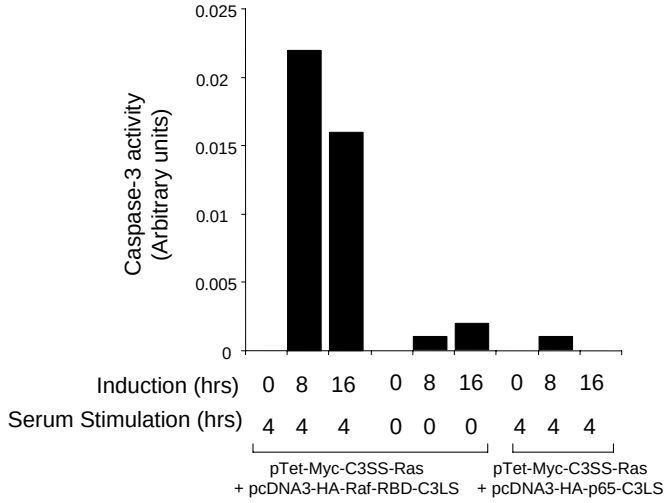

Figure 11. Serum stimulation activates caspase activity of RRC-MCAMD. PTet-Myc-C3SS-Ras or pTet-Myc-p65-C3SS was co-transfected with pcDNA3-HA-Raf-RBD-C3LS into HEK293 cells. After transfection, the cells were serum-starved, and expression of Myc-C3SS-Ras or Myc-p65-C3SS was induced by removing tetracycline in the medium for indicated time. After stimulation by 10% fetal bovine serum for 4 hours, the cells were lysed. HA-Raf-RBD-C3LS was immunoprecipitated with an anti-HA antibody and used for caspase-3 activity assay with a caspase-3 activity assay kit (BioVision). The activity of caspase-3 is in an arbitrary unit.

A4.5. The RRC-MCAMD induces apoptosis in response to mitogen stimulation. We further examined the effect of the RRC-MCAMD on cellular apoptosis upon serum stimulation in HEK293 cells. To control expression level and timing of C3SS-Ras in cells, we established a tet-off tetracycline-inducible cell line that expresses C3SS-Ras. To test the effect of the RRC-MCAMD on coupling mitogenic signal to cellular apoptosis, we transiently transfected pcDNA3-HA-Raf-RBD-C3LS into the tetracycline-inducible cell line that expresses C3SS-Ras. After 36 hrs transfection of Raf-RBD-C3LS, we began to induced expression of C3SS-Ras by removing tetracycline in the medium, followed by 4 hrs serum starvation, then 5 hrs stimulation of cells by 10% FBS. The apoptotic cells were observed under a phase microscope. As shown in Figure 12A, after co-expression of C3SS-Ras and Raf-RBD-C3LS, the cells went to apoptosis upon treatment of 10% FBS (panel d). The controls, including co-expressed C3SS-Ras and Raf-RBD-C3LS without treatment of FBS (panel

c), co-expressed C3SS-Ras and p65-C3LS with and without 10% FBS treatment (panels f and g), and expressed Raf-RBD-C3LS alone with or without 10% FBS treatment (panels a, b, and e), did not show any visible apoptotic cells. To further assess the apoptotic effect, we performed DNA ladder assay. We transfected pcDNA3-HA-Raf-RBD-C3LS into pTet-Myc-C3SS-Ras tetracycline-inducible cell line. To enhance the apoptotic signal, we induced expression of C3SS-Ras for 24 hrs after 24 hrs transfection of Raf-RBD-C3LS. During the induction time, we serum-starved the cells for 8 hrs followed by 16 hrs 10% FBS treatment. The results were shown in Figure 12B. Consistent with Figure 12A, when C3SS-Ras was co-expressed with Raf-RBD-C3LS upon 10% FBS treatment, a severe DNA fragmentation was occurred (lane 5). There was a minor DNA fragmentation occurred in the cells that co-expressed C3SS-Ras and Raf-RBD-C3LS without serum treatment (24 hrs serum starvation) (lane 6), which might be caused by a minor mitogenic process even under serum starvation condition or the long-term serum-starvation. Other controls, including expression of C3SS-Ras alone (lanes 1 and 2) and co-expression of C3SS-Ras and p65-C3LS (lanes 3 and 4), did not show significant DNA fragmentation. Taken together, these data indicate that the RRC-MCAMD is capable of coupling of mitogenesis to apoptosis.

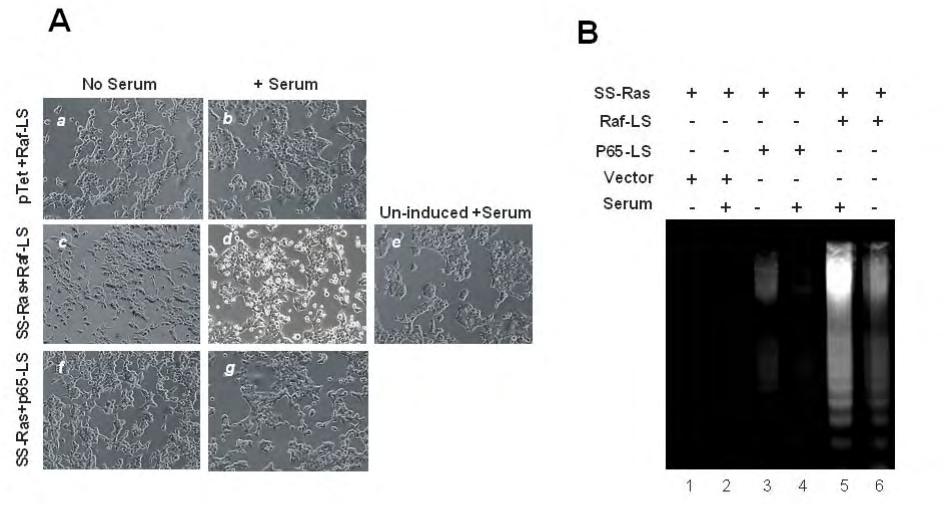

Figure 12. The RRC-MCAMD induces apoptosis in response to mitogen stimulation. A, pTet or pTet-myc-C3SS-Ras tetracycline-inducible stable cell line was transfected with pcDNA3-HA-Raf-RBD-C3LS or pcDNA3-HA-p65-C3LS. The expression of C3SS-Ras was induced after the transfection for 36 hrs. At the same time, the cells were serum-starved for 4 hours and then stimulated with or without 10% FBS for 5 hrs. The cells were monitored under an inverted phase microscope. **B,** pTet-myc-C3SS-Ras tetracycline-inducible cell line was transfected with pcDNA3, pcDNA3-HA-Raf-RBD-C3LS, or pcDNA3-HA-p65-C3LS. The expression of C3SS-Ras was induced after the transfection for 24 hrs. At the same time, the cells were serum-starved for 8 hrs and stimulated with or without 10% FBS for 16 hrs. The cells were harvested and the fragmentated DNA was extracted, resolved in 1% agarose gel by electrophoresis, and visualized by ethidium bromide staining.

3. Potential problems and future

studies.

3.1. NACAM (NF-κB activation-coupled apoptosis molecule). The major problem for creating NACAM is that homo-dimerization of NF-κB-SS and NF-κB-LS. The homo-dimerization hinders heterodimerization that is required for formation of active caspase-3 and induction of cellular apoptosis. Co-expression of NF-κB-SS and NF-κB-LS may overcome the problem. However, co-expression of NF-κB-SS/NF-κB-LS may cause immediate un-controllable activation of caspase-3. In future, we may consider co-expression of IκB to control the process. Another problem is the linker between NF-κB and caspase-3 subunits. We only made a single linker in the fusion proteins. We did not have time to try different linkers for optimal conformation for formation of effective caspase-3. We will make different length of linkers in future studies. In addition, we need to further characterize NACAM for its caspase-3 activity and induction of cellular apoptosis in response to activation of NF-κB in HEK293 cells and in prostate cancer cells.

3.2. RRC-MCAMD (Ras/Raf/Caspase-3-based mitogenesis-coupled apoptosis molecular device). In future, we will focus on construction of retrovirus vector-based RRC-MCAMD. We will test killing effect of the retrovirus vector-based RRC-MCAMD on tumor cells and xenografted tumor on mice, laying a base for application of the RRC-MCAMD to cancer gene therapy.

Key Research accomplishments

(1) We have successfully constructed 32 fusion proteins for testing NF-kB-activation-coupled apoptosis, and examined the expression of these fusion proteins.

(2) We have examined formation of NACAM (NF-kB activation-coupled apoptosis molecule) by p65-L-SS, p65-L-LS, p65-SS, p65-LS, p105-SS, and p105-LS, and detected caspase-3 activity from co-immunoprecipitation of p65-L-SS and p65-L-LS. The data suggest that p65-L-SS and p65-L-LS are capable of forming effective caspase-3. This work provides a preliminary study for creating NF-kB activation-coupled apoptosis molecule.

(3) We have detected caspase-3 activity of the RRC-MCAMD (Ras/Raf/Caspase-3-based mitogenesis-coupled apoptosis molecular device) upon stimulation by serum (mitogen). We have successfully induced cellular apoptosis by introducing the RRC-MCAMD into the cells. These data has laid foundation for application of the RRC-MCAMD to cancer therapy. Thus, we have established the RRC-MCAMD in a cell system.

Reportable outcomes

(1) Through this research, we have gained experience in creating NF-kB activation-coupled apoptosis molecule and built a solid base for future exploration of application of NACAM for prostate cancer therapy.

(2) We have established the RRC-MCAMD in a cell system, laying the base for developing the RRC-MCAMD into a prostate cancer gene therapeutic technique.

(3) We are patenting the RRC-MCAMD as a potential cancer gene therapy technique.

(4) We are preparing a manuscript regarding to the cellular signal rewiring of the RRC-MCAMD for publication.

Conclusions

(1) Our studies have demonstrated that p65-L-SS and p65-L-LS are capable of forming the NF-κB activation-coupled apoptosis molecule (NACAM), as described in the proposal. If we can improve hetero-dimerization of p65-L-SS or p65-L-LS in future, we will increase the efficiency of NACAM and effectively induce cellular apoptosis by NACAM and use NACAM in prostate cancer therapy.

(2) Our studies have established Ras/Raf/Caspase-3-based mitogenesis-coupled apoptosis molecular device (RRC-MCAMD) in a cell system and shown that RRC-MCAMD is a promising technique for cancer gene therapy. In future, we will further develop the RRC-MCAMD into a retrovirus vector-based prostate cancer gene therapeutic technique.

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Appendices

(1) A list of Personnel receiving pay from the research effort:

Wannian Yang, Ph.D., Staff Scientist (5% effort)
Fengchang Zhu, Ph.D., Postdoctoral Fellow (100% effort)